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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 1/20, 15/31, A61K 39/04 // (C12N 1/20, C12R 1:32)

A2

(11) International Publication Number: WO 99/10475

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/CA98/00790

(22) International Filing Date: 20 August 1998 (20.08.98)

(30) Priority Data:
08/915,709 21 August 1997 (21.08.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 08/915,709 (CIP)
Filed on 21 August 1997 (21.08.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ATTENUATED STRAINS OF MYCOBACTERIA

(57) Abstract

Attenuated strains of *Mycobacterium*, particularly species of the tuberculosis complex, have the mycobacterial cell entry (*mce*) gene functionally disabled. The gene may be disabled by an insertion into the gene which disrupts the mycobacterial cell entry function thereof of a selectable marker which is used for screen for homologous recombinants in which a double cross–over event has been effected. The attenuated strains may be used in the immunization of hosts against *Mycobacterium* disease.

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TITLE OF INVENTION

ATTENUATED STRAINS OF MYCOBACTERIA

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of United States Patent Application No. 08/915,709 filed August 21, 1997.

FIELD OF INVENTION

The present invention relates to the field of molecular immunology and, in particular, to attenuated strains of *Mycobacterium* and immunogenic preparations comprising the same.

BACKGROUND TO THE INVENTION

Tuberculosis (TB) is a major cause of mortality throughout the world, particularly in developing countries. There are about 8 to 9 million new cases of clinical disease reported every year and the number of deaths is estimated to be about 3 million. In the U.S. the trend of steady decline in TB has reversed and the problem is compounded by increasing numbers of drug-resistant strains. The tuberculosis complex is a group of four mycobacterial species that are genetically closely related. The three most important members are *Mycobacterium tuberculosis*, the major cause of human TB; *Mycobacterium africanum*, a major human pathogen in some populations; and *Mycobacterium bovis*, the cause of bovine TB. None of these mycobacteria is restricted in being pathogenic for a single host species.

In addition to being an important human disease, TB is also a major veterinary problem in many countries. Infection of cattle with *M. bovis* results in bovine TB and all animals showing any signs of infection are systematically slaughtered. The economic losses are thus extensive, and furthermore, cattle can serve as a reservoir for human disease.

In a majority of cases of infection, inhaled tubercle bacilli are ingested by phagocytic alveolar macrophages and are either killed or grow intracellularly to a limited extent in local lesions called tubercules. In this way the infection is limited and the primary sites of infection are walled off without any symptoms of disease being observed. Such individuals have a lifetime risk of about 10% for developing active disease. In a latter eventuality, bacilli spread from the site of infection in the lung, through the lung and via lymphatics or blood to other parts of the body producing characteristic solid caseous (cheese-like) necrosis in which bacilli survive. If the necrotic reaction expands breaking into a bronchus, or in the worst case, if the solid necrosis liquefy, a rapid proliferation of the bacilli occurs. The pathological and inflammatory processes set in motion then produce the characteristic weakness, fever, chest pain, cough and bloody sputum which are the hallmarks of active TB.

Effective treatment of TB with antibiotics exists. However, this is expensive and requires prolonged administration of a combination of drugs. There is a problem in compliance with the drug administration regime because of the extended time periods involved and this has contributed to the appearance of drug-resistant strains. There is a recognized vaccine for TB which is an attenuated form of *M. bovis*, known as BCG (bacilla Calmette Guérin). This strain was developed in 1921 and the basis for its attenuation is still not known (ref. 1 - throughout this application, various references are cited in parentheses to describe more fully the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure).

The efficacy of BCG as a TB vaccine is a subject of controversy and has been estimated in various trials to be anywhere between 0 and 70%.

The molecular basis for the virulence and pathogenesis of *M. tuberculosis* have not been extensively described. Some virulence factors, particularly those related to the sigma factors have been recently identified (ref. 2). *M. tuberculosis* can enter non-phagocytic cells in culture, such as HeLa cells (ref. 3) and once inside can multiply and survive. Recently, a protein encoded by a DNA fragment (1535 bp long) from a strain of *M. tuberculosis* (H37Ra) was reported to mediate the entry of the bacterium and its survival in mammalian cells (ref. 4). This DNA fragment when introduced into a non-pathogenic strain of *E. coli* is able to confer invasiveness to the bacterium, and survival for up to 24 hours in human macrophages. The *mce* (mycobacterial cell entry) gene was mapped to an Open Reading Frame (ORF) extending from position 182 to 810 on the 1535bp DNA fragment mentioned above and encodes a protein of molecular weight between 22 and 27 kDa. Subsequent work has shown the gene described in ref. 4 is not a full length gene.

In copending United States Patent Application No. 08/677,970 filed July 10, 1996, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference (WO 98/01559), there is described the isolation and characterisation of genes encoding proteins of mycobacteria associated with cell binding and cell entry and the protein encoded thereby. This gene is referred to herein as the Mycobacterial cell entry (*mce*) gene and the encoded protein the Mycobacterial cell entry protein (Mcep).

Mycobacterial infection may lead to serious disease. It would be advantageous to provide attenuated strains of *Mycobacterium* wherein the mycobacterial cell

entry gene is disabled, and immunogenic preparations including vaccines comprising the same.

SUMMARY OF INVENTION

The present invention provides attenuated strains of Mycobacteria which are useful in immunogenic compositions. In accordance with one aspect of the present invention, there is provided an attenuated strain of *Mycobacterium* wherein the mycobacterial cell entry (*mce*) gene is functionally disabled. By functionally disabling the *mce* gene, the ability of the *Mycobacterium* to invade and infect cells is removed. This attenuation permits the novel strains provided herein to be used in immunogenic compositions for administration to a host to generate an immune response.

The *mce* gene may be functionally disabled by an insertion into the gene such as to disrupt the mycobacterial cell entry function thereof. The *mce* gene also may be functionally disabled by deleting at least a part of the gene from the wild-type strain. In addition, mutagenesis of the *mce* gene may be used to attenuate the wild-type strain.

The mutant strain of *Mycobacterium* may be prepared by any convenient procedure. Homologous recombination conveniently may be used to replace the *mce* gene of the wild-type strain of *Mycobacterium* by a double cross-over event with a disabled *mce* gene.

The present invention is broadly applicable to strains of *Mycobacterium*, particularly a species of the tuberculosis complex, including *M. tuberculosis* and *M. bovis*.

In another aspect of the invention, there is provided a method of forming an attenuated strain of *Mycobacterium*, which comprises effecting allelic exchange of a mutant mycobacterial cell entry (*mce*) gene which is functionally disabled for a mycobacterial cell entry gene in a wild-type strain of *Mycobacterium*.

The mutant *mce* gene may contain a selectable marker, so that the attenuated strain of mycobacterium formed in the allelic exchange may be detected on the basis of the presence of the selectable marker therein.

A further aspect of the invention provides an immunogenic composition comprising the attenuated strain provided herein. Such immunogenic composition may be formulated as a vaccine for *in vivo* administration to a host to confer protection against disease caused by a virulent strain of *Mycobacterium*. The host may be a primate including a human.

The present invention includes, in a further aspect thereof, a method of generating an immune response in a host comprising administering thereto an immunoeffective amount of the immunogenic composition provided herein.

A yet further aspect of the invention provides a method of producing a vaccine for protection against a disease caused by infection by a virulent strain of *Mycobacterium*, which comprises administering the immunogenic composition provided herein to a first host to determine an amount and frequency of administration thereof to confer protection against the disease; and formulating the immunogenic composition in a form suitable for administration to a treated host in accordance with the determined amount and frequency of administration. The treated host may be a human.

The attenuated strains of Mycobacterium provided herein are useful as a live vaccine against diseases caused by Mycobacteria. Advantages of the present invention include the provision of safer and attenuated strains of *Mycobacterium* for the preparation of immunogenic compositions, including vaccines, and for the generation of immunological and diagnostic reagents.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 illustrates the construction and restriction map of a disrupted *mce* gene. The hygromycin resistance gene (*hyg*) from *S. hygroscopicus* was inserted at the *BsiWI* site in the *mce* gene. Primers P4414 (SEQ ID NO: 1) and P4448 (SEQ ID NO: 2) are located respectively 5' and 3' of the *BsiWI* site. Only the 4.7 kb insert of plasmid pBCGcepX and the 6 kb insert of plasmid pBCGcepX-H are represented. The *Sac*I site of plasmid pBlueScript SK+ from which these plasmids are derived is located in the multiple cloning site in 5' of the *Xho*I site. Digestion of plasmid pBCGcepX with *Sac*I yields two bands (~3.75 kb and ~3.9 kb). Digestion of plasmid pBCGcepX-H with *Sac*I yields three bands (~1.66 kb, ~3.45 kb and ~3.9 kb). The *Apa*I site of plasmid pBlueScript SK+ is located in the multiple cloning site 3' of the *Xho*I site. Probe PMCE is represented by the bold arrow and covers the totality of the *mce* gene.

Figure 2 contains a computer scan illustrating screening of hygromycin-resistant BCG colonies by PCR to detect double cross-over events in homologous recombination. The PCRs were performed on BCG colonies with primers P4414 and P4448. The expected amplification product is 572 bp for the wild-type (lane wt) and about 1.9 kb in case of a double cross-over event (lanes 65 and 73). In case of a single cross-over event or a non-specific integration, the presence of the two amplification products was expected. Lane neg: negative control, no DNA. Lane wt: BCG wild-type. A 572 bp fragment was amplified. Lane 69: BCG-69. Integration of plasmid pBCGcepX-H in the chromosome was the result of a single cross-over event, or a non-specific integration. The amplification reaction yields two products. Lane 65 and 73: BCG-65 and BCG-73. A double cross-over event lead to the integration

of the mutated *mce* gene in the chromosome. A 1.9 kb fragment was amplified.

Figure 3 contains a computer scan of a Southern Blot analysis of chromosomal DNA from BCG strains.

Lane 1: BCG wild-type)
Lane 2: BCG-65 (⇒ digested by *Sac*I
Lane 3: BCG-69)

Lane 4: BCG wild-type)
Lane 5: BCG-65 (
Lane 6: BCG-69) ⇒ digested by *Xho*I

Lane 7: BCG-73 (
Lane 8: BCG-83)

For the XhoI digests, the wild-type strain gave a single band at 4.7 kb while the mutants gave a band at 6 kb, resulting from the presence of the *hyg* gene. BCG-69 gave two bands, confirming the presence of the wild-type copy of the gene as well as the disrupted one in the chromosome. While the wild-type mutant gave the 5.2 kb band for the *Sac*I digest, the knock-out mutant, BCG-65, gave two bands at 4.8 kb and 1.7 kb resulting from the *Sac*I site of the *hyg* gene integrated into the *mce* gene.

Figure 4 contains a computer scan of a Western Blot analysis of Mcep produced by mutants.

Lane 1: BCG-65
Lane 2: BCG-69
Lane 3: BCG wild-type

A monoclonal antibody against Mcep was used for the blotting. Mcep is not present in BCG-65, while produced by BCG-69 or BCG wild-type.

Figure 5 is a bar graph illustrating the difference in the ability of BCG_{mce}^- to invade HeLa cells compared to BCG wild-type.

Figures 6 to 9 contain graphical representatives of the growth of BCG wild-type (wt) or knock-out mutant BCG-65 (KO) in the organs of CB17-SCID mice. Figure 6 shows the results for livers, Figure 7 for lungs, Figure 8 for spleens and Figure 9 for kidneys.

GENERAL DESCRIPTION OF THE INVENTION

The use of BCG herein is a useful means of illustrating the broader application of the present invention to functionally disabling the mycobacterial cell entry gene in a strain of *Mycobacterium*, including any of the species of the tuberculosis complex, including *Mycobacterium tuberculosis*. The provision of the strain of *Mycobacterium* in which the *mce* gene is functionally disabled provides attenuated strains of *Mycobacterium* which may be used safely in immunogenic compositions.

Referring to Figure 1, there is illustrated therein the construction of a disrupted *mce* gene. Plasmid pBCGcepX, the preparation of which is described in the above mentioned US Application No. 08/677,970 and deposited under ATCC No. 97511, is digested with restriction enzyme *BsiWI* to cut the *mce* gene at the restriction site. In Figure 1 only the 4.7 kb *Xho*I fragment of the plasmid is shown.

The hygromycin resistance gene (*hyg*) of *Streptomyces hygroscopices* is isolated from a plasmid pIDV6, obtained from ID Vaccines, by digestion with restriction enzyme *Not*I. Following separation of a 2.5 to 3 kb fragment, restriction enzyme *BspHI* is used to isolate a 1.3 kb fragment containing *hyg* gene.

The *hyg* gene is ligated with the *BsiWI* digested plasmid pBCGcepX and the ligate used to transform *E. coli*. Following selection for hygromycin resistance, transformants are grown and the plasmid isolated. Plasmid pBCGcepX-H, produced by this procedure, has the *hyg* gene inserted into the *mce* gene, in the opposite direction.

The plasmid BCGcepX-H is linearized and the linearized plasmid is used to transform a *Mycobacterium* strain, for example, *M. bovis* BCG, by homologous recombination. The construction by homologous recombination of mutants deficient in some metabolic

genes has been achieved recently in slow growing mycobacteria (refs. 5, 6, 7). The suppression of key metabolic enzymes was expected to lead to the generation of less virulent strains, with little success so far (ref. 8).

Screening of recombinant events may be performed by PCR analysis. Hygromycin resistant *M. bovis* BCG colonies are subjected to PCR analysis using a pair of primers corresponding to appropriate portions of the *mce* gene. As seen in Figure 1, primer P4414 (SEQ ID NO: 1) and P4448 (SEQ ID NO:2) (the nucleic acid sequences of the primers are shown in Table 1 below), are used for PCR amplification. Such primers generate a 572 bp PCR product from a wild-type strain while integration of the mutant *mce* gene by homologous recombination with double cross-over yields a 1.9 kb product. For a random DNA integrate or a single cross-over, two fragments are amplified.

Three mutants (BCG-65, BCG-73, BCG-83) produced only a 1.9 kD PCR-amplified fragment, consistent with homologous recombination causing replacement of native *mce* gene by a disrupted copy of the gene. Figure 2 shows the results of the PCR analysis. The wild-type strain produced a 572 bp fragment while a single cross-over mutant produced both fragments.

In order to further assess the recombinant BCG as to the proper integration of the functionally-disabled *mce* gene, a Southern blot was performed. This required isolating the chromosomal DNA from the recombinant BCG colonies and digesting them with restriction endonucleases, and transferring the DNA fragments separated on the agarose gel to a nylon membrane. The probe for the *mce* gene was PCR amplified from *M. tuberculosis* H37RV DNA as described in Example 6 below. The 1.6 kb probe was used to verify the double cross-over events that occurred in BCG-65, BCG-73 and BCG-83.

These strains represent attenuated BCG containing the functionally disrupted *mce* gene.

To show that these attenuated BCG no longer produce the cell entry protein, Western blots were performed on cell lysates produced by sonication of the cells. A mycobacterial strain with a disrupted gene would not be able to make the Mce protein and, therefore, a mouse monoclonal antibody to the mycobacterial cell entry protein would not recognize any protein from this strain, as described in Example 7 below. Figure 4, lane 1, clearly shows that such attenuated BCG, BCG-65, does not make any mycobacterial cell entry protein. A single cross-over or non-homologous recombinant, BCG-69, was not disrupted in the *mce* gene and still produced the wild-type mycobacterial cell entry protein (Fig. 4, lane 2).

Biological Deposits

A vector that contains the gene encoding a mycobacterial cell entry protein and having a molecular weight of between about 45,000 and about 60,000 from the *M. bovis* strain BCG that is described and referred to herein has been deposited with the American Type Culture Collection (ATCC) located at 12301 Parklawn Drive, Rockville, Maryland 20852, USA, pursuant to the Budapest Treaty and prior to the filing of this application in connection with Application No. 08/677,970 referred to above. Samples of the deposited vectors will become available to the public upon grant of a patent based upon this or the aforementioned United States patent application and all restrictions on access to the deposit will be removed at that time. Viable samples will be provided if the depository is unable to dispense the same. The invention described and claimed herein is not to be limited in scope by the biological material deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent or similar vectors that encode similar or

equivalent antigens as described in this application are within the scope of the invention.

Deposit Summary

Deposit	ATCC Designation	Date Deposited
Plasmid pBCGcepX	97511	April 11, 1996

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Example 1:

This Example illustrates the recombinant DNA methods used herein.

Restriction enzymes and cloning vectors were obtained from several sources including New England Biolabs, Life Technologies, Boehringer Mannheim and Stratagene. The enzymes and buffers for the PCR were purchased from Perkin-Elmer or Sangon Corporation and used as per the manufacturers protocols.

Reagents used in DNA isolation protocols were purchased from Sigma Biochemicals. Most recombinant DNA manipulations were performed using standard protocols (ref. 10). Sequences of double stranded plasmid DNA were determined using the Taq Dye Deoxy

Terminator cycle sequencing kit (Applied Biosystems) on a GeneAmp PCR system 9600 (Perkin-Elmer) and a run on a DNA analysis system, model 370A (Applied Biosystems). The sequence was assembled and processed using the IG software (IntelliGenetics Inc). The synthesis of oligonucleotides used as primers was performed using an Applied Biosystems (380B) synthetizer. The synthetic oligonucleotides were purified on OPC cartridges supplied by Applied Biosystems according to the manufacturers protocol.

Example 2:

This Example illustrates construction of the disrupted *mce* gene.

5 μg of plasmid pBCGcepX (ATCC # 97511) were digested with restriction enzyme *BsiWI* (NEB Biolabs) for 2 hours at 37°C in 25 μl final volume. 3 μl of Nick translation buffer, 1 μl of dNTP's (2mM) and 2 units of Klenow DNA Polymerase (Boehringer Mannheim) were added to the solution and it was incubated for 30 min at room temperature. 120 μl of water were added and a phenol-chloroform extraction was performed by mixing: 75 μl of phenol (Life Technologies) and 75 μl of chloroform-isoamyl acid (24: 1, v:v) to the solution. The tube was spun (12000 xg for 2 min) and the aqueous phase was transferred to a fresh tube. 300 μl of ice-cold 100% ethanol was added, the DNA was pelleted by centrifugation (12000 g for 15 min at 4°C), and washed with 1 ml of 70 % ethanol. The DNA was air dried at room temperature and resuspended in 40 μl of water. 3 units of Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim) were added and the mixture was incubated at 37°C for 1 hour in 50 μl volume final. The DNA was purified from an agarose gel, and resuspended in 30 μl of water.

To isolate the hygromycin resistance gene (*hyg*) of *Streptomyces hygroscopicus*, 18 µg of plasmid pIDV6 (obtained from Dr Horwitz, University of California, Los Angeles, CA) were digested with the restriction enzyme *Not*I (NEB Biolabs) for 3 hours at 37°C in 60 µl volume final. The digestion of plasmid pIDV6 with *Not*I resulted in two products, namely a 2.5 to 3 kb fragment containing the *hyg* gene and a larger fragment. The 2.5 kb band was purified and resuspended in 20 µl of water. The restriction enzyme *BspH*I (NEB Biolabs) was added to the DNA and the mixture was incubated at 37°C for 2 hours 30 min, in 30 µl final volume. 3,5 µl of Nick translation buffer, 1 µl of dNTP's (2mM) and 2 units of Klenow DNA Polymerase (Bochringer Mannheim) were added to the solution and the mixture was incubated for 30 min at room temperature. The digest was run on a 0,8% agarose gel, and consisted in two products, namely a 1.3 kb fragment and a smaller one. The larger piece of DNA, containing the *hyg* gene, was purified from the gel and resuspended in 15 µl of water.

The ligation was performed in a final volume of 20 µl, using 1 µl of plasmid pBCGcepX digested by *BsiW*I and treated as described above and 4 µl of the *hyg* gene isolated as described above. 1.5 units of T4 DNA Ligase (Life Technologies) were used in this reaction. The mixture was incubated overnight at 16°C to ligate the *hyg* gene with the digested pBCGcepX plasmid.

2 µl of the ligation mixture were used to transform 70 µl of electro-competent *E. coli* HB101 cells, and 100 µl of the transformation solution were plated onto Luria-Bertani agar (LB agar), with 100 µg/ml of ampicillin and 200 µg/ml of hygromycin B (Boehringer Mannheim). A few transformants were isolated and grown up. The plasmids were isolated using a kit for high grade plasmid purification (Qiagen) and sequenced. One

of them, plasmid pBCGcepX-H, had the *hyg* gene inserted in the *mce* gene, in the opposite direction (see Fig. 1). 50 μg of plasmid pBCGcepX-H were digested with the restriction enzyme *ApaI* (Life Technologies) for 3 hours at 30°C in 200 μl final volume. After incubation, 100 μl of water were added and the DNA was purified by phenol extraction, followed by two phenol-chloroform extractions. The aqueous solution was transferred to a new tube, 35 μl of 3M sodium acetate were added, the DNA was precipitated by adding 1 ml of ice-cold 100% ethanol. The DNA was pelleted by centrifugation (12000 g for 10 min at 4°C), washed with 70% ethanol, air dried and resuspended in 25 μl of water. The concentration of DNA was determined by reading the OD at 260nm.

Example 3:

This Example illustrates transformation of *M. bovis* BCG with plasmid pBCGcepX-H

Electrocompetent *M. bovis* BCG cells were prepared using a modification of a protocol already described (ref. 9). 500 μl of a frozen stock of Connaught *M. bovis* BCG strain were used to inoculate 10 ml of 7H9-ADC-Tw broth and incubated with shaking at 37°C for three days. Two ml of this preculture were used to inoculate 100 ml of 7H9-ADC-Tw broth and incubated at 37°C with shaking for three days. 1.5 g of glycine (Boehringer Mannheim) diluted in 10 ml of water and sterile-filtered was added to the culture and the culture was incubated one more day.

The electrocompetent cells were spun down (4000 g for 15 min) and sequentially washed in 100, 50, 25, 10 ml of 10% glycerol. The cells were eventually resuspended in 3 ml of 10% glycerol.

A 0.25 ml aliquot of resuspended cells was mixed with 3 μg of linearized plasmid pBCGcepX-H, the mixture was incubated on ice for 10 min and subjected to electroporation in a 0.2 cm cuvette using a BioRad

apparatus (BioRad,) at a setting of 2,5 kV, capacitance of 25 µF and pulse controller to 1000 Ω. The cells were then placed on ice for 10 min, resuspended in 1 ml M-ADC-TW broth and incubated for 3 hours with shaking at 37°C. The transformed cultures were spread on 7H10 agar plates containing 50 µg/ml of hygromycin B and 100 µg/ml of cycloheximide(Sigma) and incubated at 37°C for 3 to 4 weeks.

Example 4:

This Example illustrates PCR amplification of the *M. bovis* BCG colonies.

Screening of recombinant events was performed by PCR reactions. Hygromycin-resistant *M. bovis* BCG colonies, prepared as described in Example 3, were isolated, used to inoculate 3 ml of 7H9-ADC-Tw broth, and incubated for three days at 37°C. 1 ml of this culture was transferred to a microfuge tube, and spun down (12000 g for 10 min) to pellet the cells. The cells were resuspended in 50 µl of water, boiled for 10 min and immediately placed on ice. The amplification reactions were carried out using the "Hot Start" procedure. Essentially, a 40 µl reaction mix containing dNTP's (0.2 mM in 100 µl final volume), buffer and a pair of primers (P4414, SEQ ID NO:1, and P4448, SEQ ID NO:2; 100 pM of each, see Table 1 below for identification of the primers) was prepared in thin-wall Eppendorf tubes. To each tube, a bead of wax (PCRGem 100, Perkin-Elmer) was added and the tube was heated to 70°C for 5 min. Subsequently, the tube was cooled at room temperature for 5 min and a reaction mix (60 µl) containing buffer, 1 unit of enzyme and 25 µl of the colony preparation was added. The tubes were then placed in a Perkin-Elmer Cetus thermal cycler and a cycling sequence started based on the following parameters:

Step 1: 2 min at 99°C;

Step 2: 45 sec at 98°C; 45 sec at 60°C; 1 min 30 sec at 72°C; repeated for 25 cycles;

Step 3: 10 min at 72°C;

Step 4: maintain at 4°C.

The tubes were stored at 4°C; aliquots of 10 µl were run on a 0,8% agarose gel and the electrophoretic patterns visualized and photographed.

The set of primers used generated a 572 bp PCR product for wild type BCG strain, while integration by homologous recombination with double cross-over yielded a 1.9 kb product. If the DNA integrated randomly or by a single cross-over, then two fragments were amplified. Analysis of 88 transformants by PCR analysis showed three mutants (BCG-65, -73, -83) yielding only a 1.9 kb fragment, as expected from homologous recombination causing replacement of the native *mce* gene by a disrupted copy of the gene. The 1.9 kb and 572 bp fragments were amplified for all the other transformants.

Figure 2 shows the results of the PCR screening described above. As may be seen therein, the mutant strain wherein a double cross-over event has caused replacement of the native *mce* gene by a disrupted form of the gene contained a 1.9 kb fragement (lanes 65, 73). The wild-type strain contained the 572 bp fragment (lane wt) while a random-integrate or a single cross-over mutant contained both the 572 bp fragment and the 1.9 kb fragment (lane 69).

Example 5:

This Example illustrates the preparation of genomic DNA from *M. bovis* BCG

Genomic DNA from BCG cultures was extracted using a modification of a technique already described (ref. 11). 50 ml of a 14 days BCG culture was centrifuged (6000 g for 10 min) to pellet the cells. The pellet was

incubated for one hour at 37°C in 1 ml of TE buffer (10 mM Tris-HCl, pH7.5 and 1mM EDTA) containing 200 µg/ml of proteinase K (Life Technologies) and 10 µg/ml of hen egg-white lysozyme (Sigma, St Louis, MO, USA). After centrifugation (12000 g for 5 min), the pellet was resuspended in 1 ml of DNAzol (Life Technologies), transferred to a 2 ml screw-capped tube filled to a quarter with glass beads (106 µm or finer, Sigma) and vortexed vigourously for 10 min. The beads were allowed to settle and the supernatant was transferred to a fresh tube and centrifuged for 10 min at room temperature. The resultant lysate was transferred to a new tube and the DNA was precipitated by adding 0.5 ml of 100% ethanol. The tube was inverted several times to mix the materials and the mixture was incubated at room temperature for 3 to 5 min. The tube was spun (at 1000g for 2 min) to pellet the DNA, the supernatant discarded, the pellet washed twice with 1 ml of 95% ethanol, air-dried at room temperature and resuspended in 200 µl of TE buffer. The quantity of DNA was estimated by measuring the optical density (OD) at 260nm in a spectrophotometer. This protocol yielded approximately 80 µg of DNA.

Example 6:

This Example illustrates the preparation of the DIG-labelled *mce* probe and Southern hybridization of BCG DNA digests.

PCR reactions were carried out on 500 ng of *M. tuberculosis* H37Rv DNA, using primers P4973 (SEQ ID NO:3) and P4974 (SEQ ID NO:4), located at the extremities of the *mce* gene of *M. bovis* BCG. PCR reactions were carried out as described in Example 4, except that the template was 500 ng of *M. tuberculosis* H37Rv DNA instead of 25 µl of a colony DNA preparation. The amplification product (1.6 kb) was isolated by excising the band from a 0.8% agarose gel and extracting the DNA. The isolated DNA was labelled with DIG-dUTP,

using the DIG-labelling kit (Boehringer-Mannheim), following the supplier's instructions. This procedure yielded the probe identified herein as PMCE. The sequence of the *mce* gene of *M. tuberculosis* H37Rv is 99% identical to the *mce* gene of *M. bovis* BCG.

8 μg of BCG DNA was digested in a 40 μl final volume, for 3 hours at 37°C, with *Sac*I or *Xho*I restrictions enzymes. The digests were run out on a 0.8% agarose gel. The gel was transferred to a nylon membrane (GeneScreen Plus, Dupont) using standard reagents and protocols and the DNA fixed to the membrane.

The membrane was prehybridized, hybridized overnight at 65°C with the labelled probe PMCE and subsequently washed. The membrane was processed following the instructions of the kit supplier (Boehringer Mannheim). The blot was exposed to a film for 3 min at room temperature and the radiograph developed (see Figure 3).

Figure 3 shows the results of the Southern Blot analysis performed as described above. Lanes 1 to 3 show the results for the *Sac*I digests. The wild-type strain (Lane 1) gave a single band as 5.2 kb while the knock-out mutant BCG-65 (Lane 2) gave two bands at 4.8 kb and 1.7 kb resulting from the *Sac*I site of the *hyg* gene integrated into the *mce* gene. The single cross-over mutant BCG-69 (Lane 3) gave three bands.

Lanes 4 to 8 show the results of the *Xho*I digests. The wild-type strain (Lane 4) gave a single band at 4.7 kb while the knock-out mutants BCG-65 (Lane 5), BCG-73 (Lane 7) and BCG-83 (Lane 8) gave a single band at 6 kb resulting from the presence of the *hyg* gene. The single cross-over mutant BCG-69 (Lane 6) gave two bands.

<u>Example 7:</u>

This Example illustrates Western Blot analysis of the BCG transformants.

M. bovis BCG transformants, prepared as described in Example 3, were grown in 10 ml of 7H9-ADC-Tw with 50 μg/ml of hygromycin, to an optical density at 600nm of 2. 1.5 ml of the culture was harvested, spun down (12000 xg for 10 min) and transformants resuspended in 200 μl of water. The solution was submitted to two 30 sec sonication cycles in a Sonifer 250 sonicator (Branson) at full power. The lysate was mixed with 4 x UMS buffer (0.1 M Tris-HCL, pH8; 20% glycerol; 8% SDS; 48% urea, 8% β-mercaptoethanol; trace of bromophenol blue). 8 μl of the mixture was boiled for 10 min, resolved on a 12.5% acrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membrane was processed using the Western Blotting system from Boehringer Mannheim, following the manufacturer's instructions. Mouse monoclonal antibodies against the mycobacterial cell entry protein (Mcep) were used for the blotting at a concentration of 1 μg/ml. The anti-mouse horseraddish peroxydase-conjugated secondary antibody (Bochringer Mannheim) was used according to the supplier's recommendations. The blot was exposed to a film for 5 min at room temperature and the auto radiograph developed (see Fig. 4).

Figure 4 shows the Western Blot results. The monoclonal antibody to Mcep detected no production of Mcep by the knock-out mutant BCG-65 (Lane 1) while production of Mcep by both the single cross-over mutant BCG-69 (Lane 2) and wild-type (Lane 3) was detected.

Example 8:

This Example illustrates an invasion assay in HeLa cells.

The invasion assay was carried out according to a method similar to that described by Isberg and Falkow (refs. 12 and 13). Bacterial samples (10^6 bacteria/well containing 10^5 cells) were added to the HeLa monolayers

in a 24-wells plate. Tissue culture plates were incubated for two hours at 37°C in a 5% CO_2 incubator. Monolayers were then washed three times with HBSS, 1 ml of cDMEM containing 100 µg/ml of amikacin was added and the plates were incubated for 1 hour at 37°C in 5% CO_2 atmosphere. After three washes with HBSS, the viable intracellular bacteria were released by lysis of the monolayers with sterile water containing 1% Tween 80 and quantitated by plating serial dilutions onto Middlebrook 7H10 agar. The viability of the HeLa monolayer was checked by Trypan Blue exclusion before lysis and more than 95% of the cells were found alive.

Figure 5 illustrates the difference in the ability of BCG_{mce}^{-} to invade HeLa cells compared to BCG wild-type. As shown in Figure 5, there is a reduction of 40% in the ability of the knock-out mutant BCG-65 (which will be referred as BCG_{mce}^{-}) to invade HeLa cells compared to that of BCG wild-type. Results are expressed as the percentage of the initial inoculum that invaded HeLa cells, and represent the mean result of two separate experiments, each performed in triplicate. The difference is statistically significant ($p<0.005$, Student t-test).

Example 9:

This Example illustrates the preparation of bacterial stocks and immunization in animals.

M bovis BCG Connaught (clinical lot of BCG IT obtained from Pasteur Mérieux Connaught) and BCG_{mce}^{-} were grown as dispersed cultures in Proskauer and Beck (PB) medium, containing 0.01% Tween 80 (PBT) and frozen in aliquots for use in experiments. For each experiment, bacteria were prepared for inoculation by thawing a vial of working stock, diluting it 10-fold in saline containing 0.05% Tween 80 and subjecting the suspension to sonication for 10s to break up clumps. The resulting suspension were diluted to the desired concentration in saline containing 0.05% Tween 80 and

injected intravenously (i.v.) in a 0.2-ml volume *via* a lateral tail vein.

CB17-SCID mice were obtained from Taconic and Charles River and were used in experiments at 6 to 8 weeks of age. They were injected i.v. with 2 x 10^5 BCG or BCG_{mce}^-. They were sacrificed at regular intervals (24h and 3, 10, 30, 60 days). One group of animals was studied for long term survival and the mice were sacrificed when found to be moribund or ill.

Example 10:

This Example illustrates the colony forming units counts in organs.

Bacteria were enumerated in the livers, spleens, kidneys and lungs of mice infected according to Example 9 by plating serial 10-fold dilutions of organ homogenates on Middlebrook 7H11 agar supplemented with ADC and glycerol, and incubating the plates for two weeks at 37°C.

Figures 6 to 9 represent the growth curves of BCG wild-type or BCG_{mce}^- in the organs of CB17 and CB17-SCID mice. As seen in Figures 6 to 9, there is no significative difference of growth of the two strains in the organs of immunocompetent CB17 mice which are able to control BCG infection. In the immunodeficient CB17-SCID mice, BCG grows in the different organs and leads to a disseminated infection and death of the animal. CB17-SCID mice inoculated with the wild-type BCG look moribund and were sacrificed at day 73 (one mouse died at day 73). CB17-SCID mice inoculated with BCG_{mce}^- were sacrificed at day 102, and although they looked sick, they were not moribund.

The difference in the number of cfus in the different organs coupled with the extended survival time of the CB17-SCID mice suggests that BCG_{mce}^- is attenuated compared to the wild-type BCG.

SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides mutants of *Mycobacterium* strains in which the expression of the mycobacterial cell entry protein is disabled. Modifications are possible within the scope of the invention.

TABLE 1: Sequence of PCR Primers

PRIMER #	SEQUENCE (5'-3')	SEQ ID NO
P4414	GTATGTGTCGTTGACCACGCC	1
P4448	TCAGGTCGATCGGCATCGTAGAAG	2
P4973	TTTCAAACGTTCCTGCGTCCC	3
P4974	CGAGTTTGACGATTCCAG	4

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CLAIMS

What we claim is:

1. An attenuated strain of *Mycobacterium* wherein the mycobacterial cell entry (*mce*) gene is functionally disabled.

2. The strain of *Mycobacterium* of claim 1 wherein said *mce* gene is functionally disabled by an insertion into the gene such as to disrupt the mycobacterial cell entry function thereof.

3. The strain of claim 2 wherein said insertion introduces a selectable marker to said *mce* gene.

4. The strain of claim 2 wherein said *mce* gene is functionally disabled by deletion of at least part of the gene from the strain of *Mycobacterium*.

5. The strain of claim 2 wherein said *mce* gene is functionally disabled by mutagenesis thereof.

6. The strain of claim 1 prepared by homologous recombination.

7. The strain of claim 1 wherein said strain of *Mycobacterium* is a species of the tuberculosis complex.

8. The strain of claim 7 wherein said strain of *Mycobacterium* is a strain of *Mycobacterium tuberculosis*.

9. The strain of claim 1 wherein said strain of *Mycobacterium* is a strain of *Mycobacterium bovis*.

10. A method of forming an attenuated strain of *Mycobacterium*, which comprises:

effecting allelic exchange of a mutant mycobacterial cell entry (*mce*) gene which is functionally disabled for a mycobacterial cell entry gene in a wild-type strain of *Mycobacterium*.

11. The method of claim 10 wherein said mutant *mce* gene contains a selectable marker and attenuated strains of *Mycobacterium* formed in said allelic exchange are detected on the basis of the presence of the selectable marker therein.

12. The method of claim 10 wherein said wild-type strain of *Mycobacterium* is a species of the tuberculosis complex.
13. The method of claim 12 wherein said wild-type strain of *Mycobacterium* is a strain of *Mycobacterium tuberculosis*.
14. The method of claim 12 wherein said wild-type strain of *Mycobacterium* is a strain of *Mycobacterium bovis*.
15. An immunogenic composition comprising the attenuated strain of claim 1.
16. The immunogenic composition of claim 15 formulated as a vaccine for *in vivo* administration to a host to confer protection against disease caused by a virulent strain of *Mycobacterium*.
17. The immunogenic composition of claim 16 wherein said virulent strain of *Mycobacterium* is a species of the tuberculosis complex.
18. The immunogenic composition of claim 17 wherein said virulent strain of *Mycobacterium* is a strain of *Mycobacterium tuberculosis*.
19. The immunogenic composition of claim 17 wherein said virulent strain of *Mycobacterium* is a strain of *Mycobacterium bovis*.
20. The immunogenic composition of claim 15 wherein said host is a primate.
21. The immunogenic composition of claim 16 wherein said primate is a human.
22. A method of generating an immune response in a host comprising administering thereto an immunoeffective amount of the immunogenic composition of claim 15.
23. The method of claim 22 wherein said immunogenic composition is formulated as a vaccine for *in vivo* administration to a host to confer protection against disease caused by a virulent strain of *Mycobacterium*.

24. The method of claim 23 wherein said virulent strain of *Mycobacterium* is a species of the tuberculosis complex.

25. The method of claim 24 wherein said virulent strain of *Mycobacterium* is a strain of *Mycobacterium tuberculosis*.

26. The method of claim 24 wherein said virulent strain of *Mycobacterium* is a strain of *Mycobacterium bovis*.

27. The method of claim 22 wherein said host is a primate.

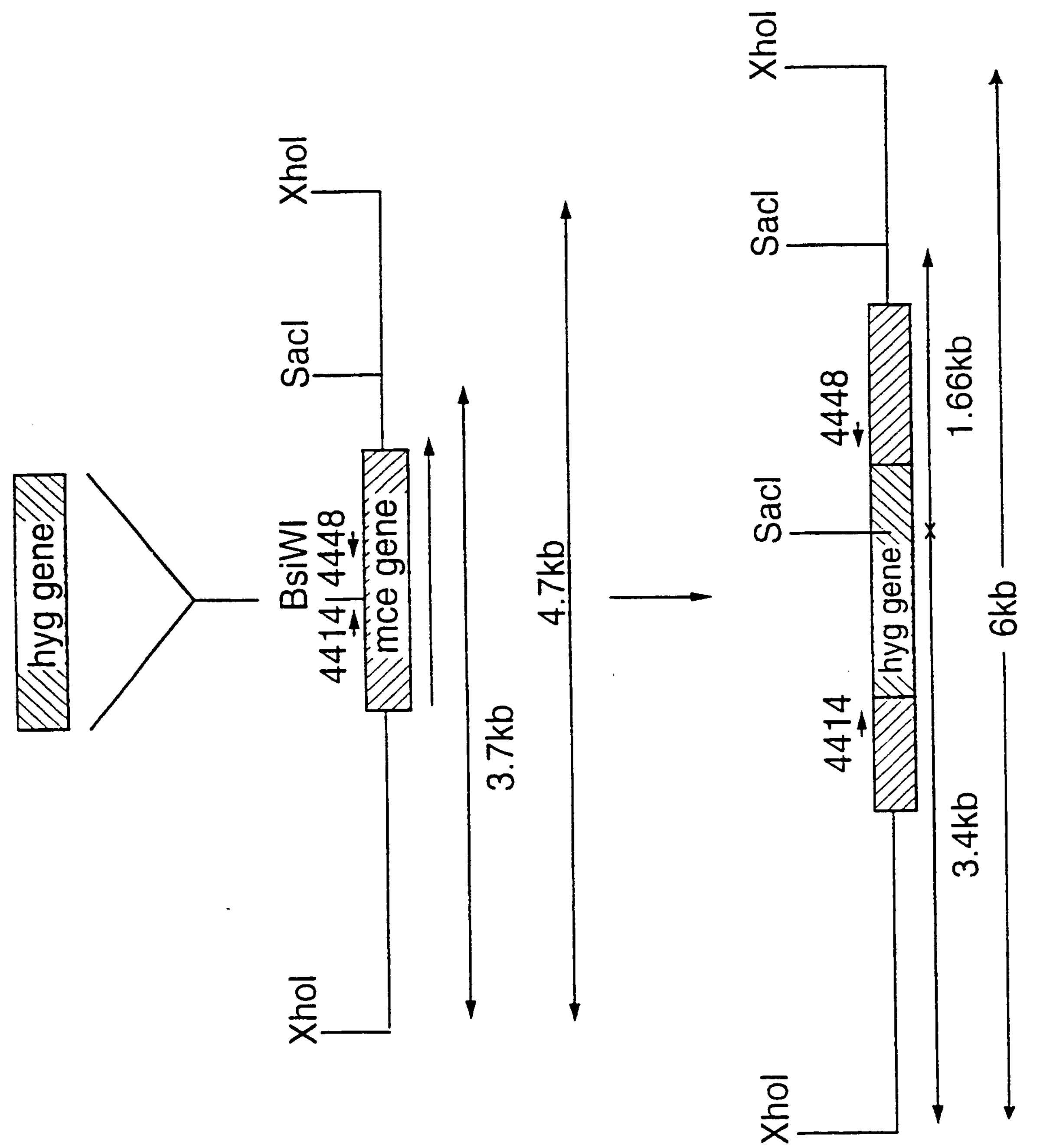
28. The method of claim 23 wherein said primate is a human.

29. A method of producing a vaccine for protection against a disease caused by infection by a virulent strain of *Mycobacterium*, which comprises:

administering the immunogenic composition of claim 15 to a first host to determine an amount and frequency of administration thereof to confer protection against the diseases; and

formulating the immunogenic composition in a form suitable for administration to a treated host in accordance with said determined amount and frequency of administration.

30. The method of claim 29 wherein the treated host is a human.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BsiWI
4414
4448
mce gene
XhoI
SacI
XhoI
3.7kb
4.7kb
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4414
XhoI
1.66kb
6kb
3.4kb

FIG.1

FIG.2

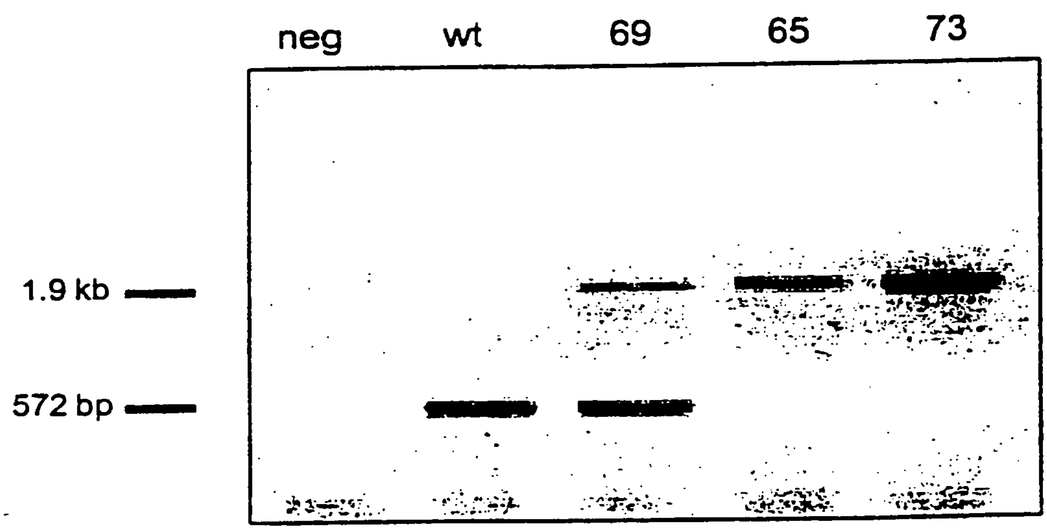


FIG.3

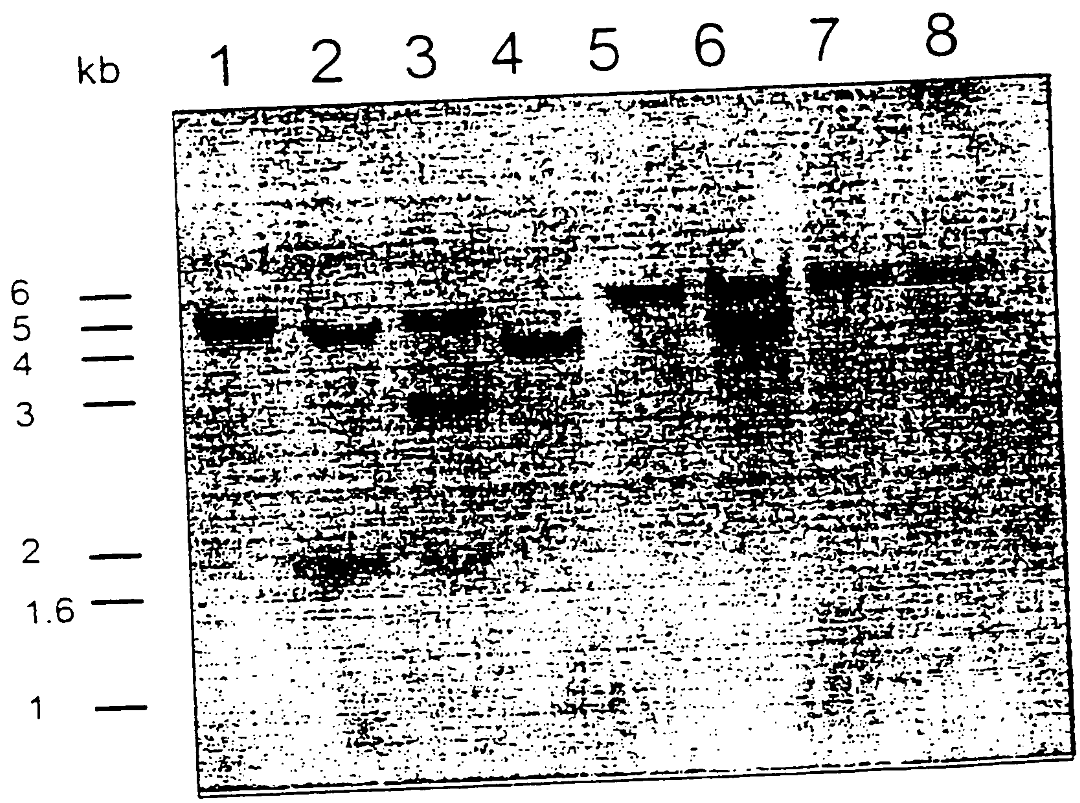
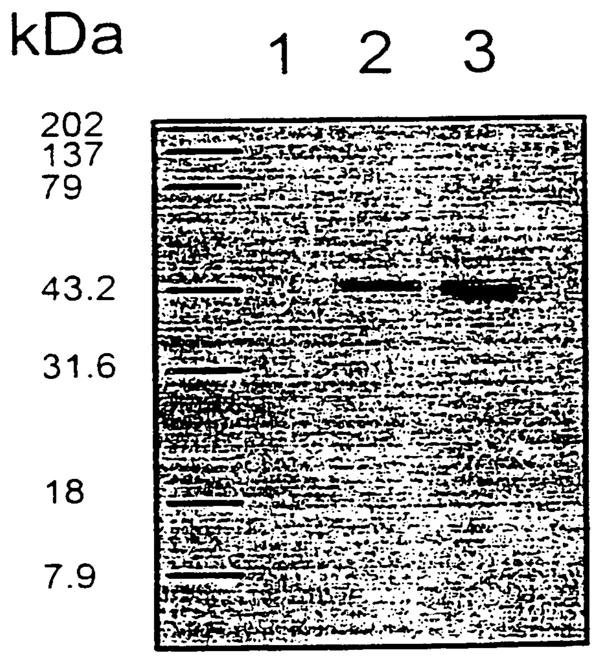


FIG.4



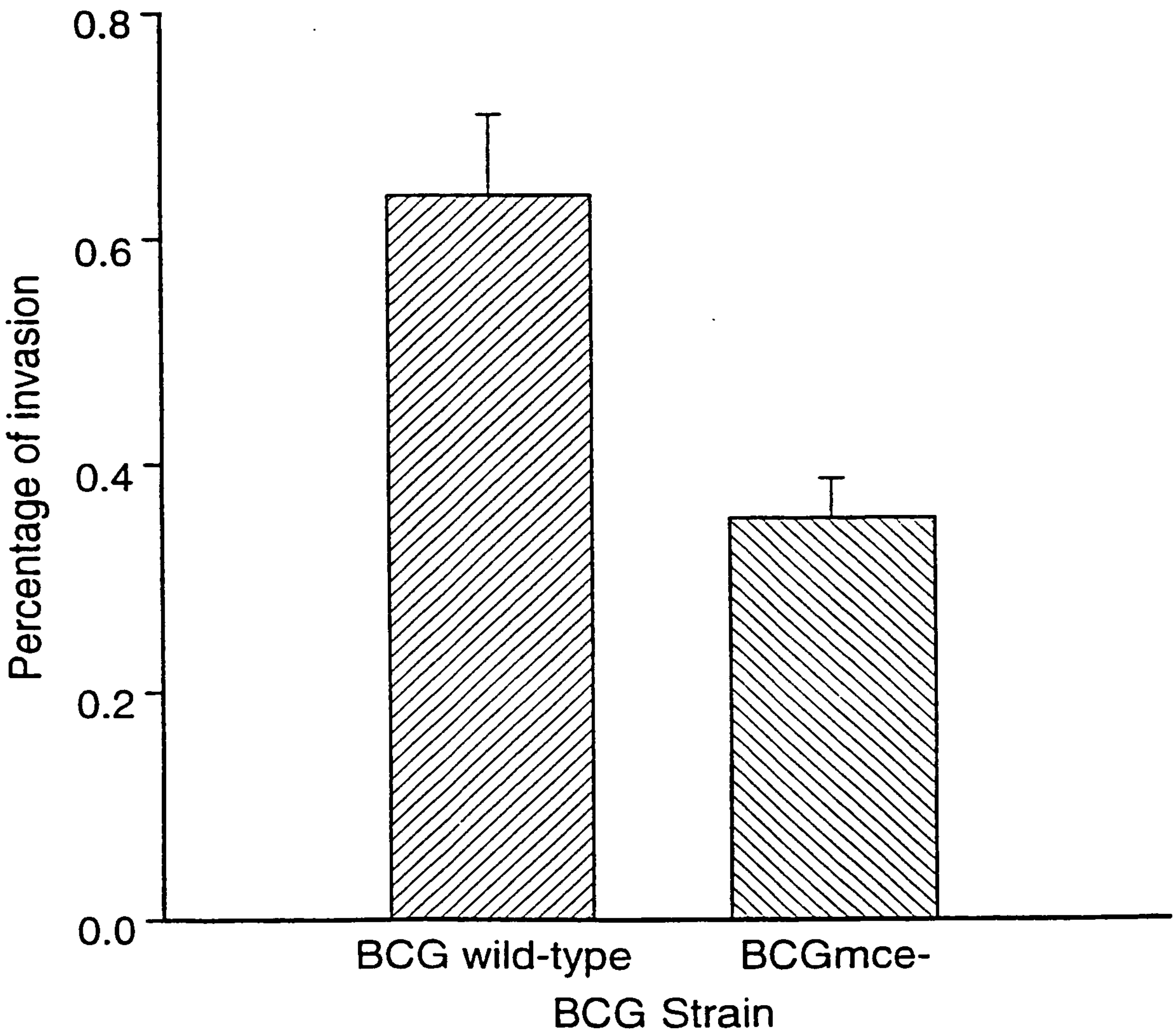
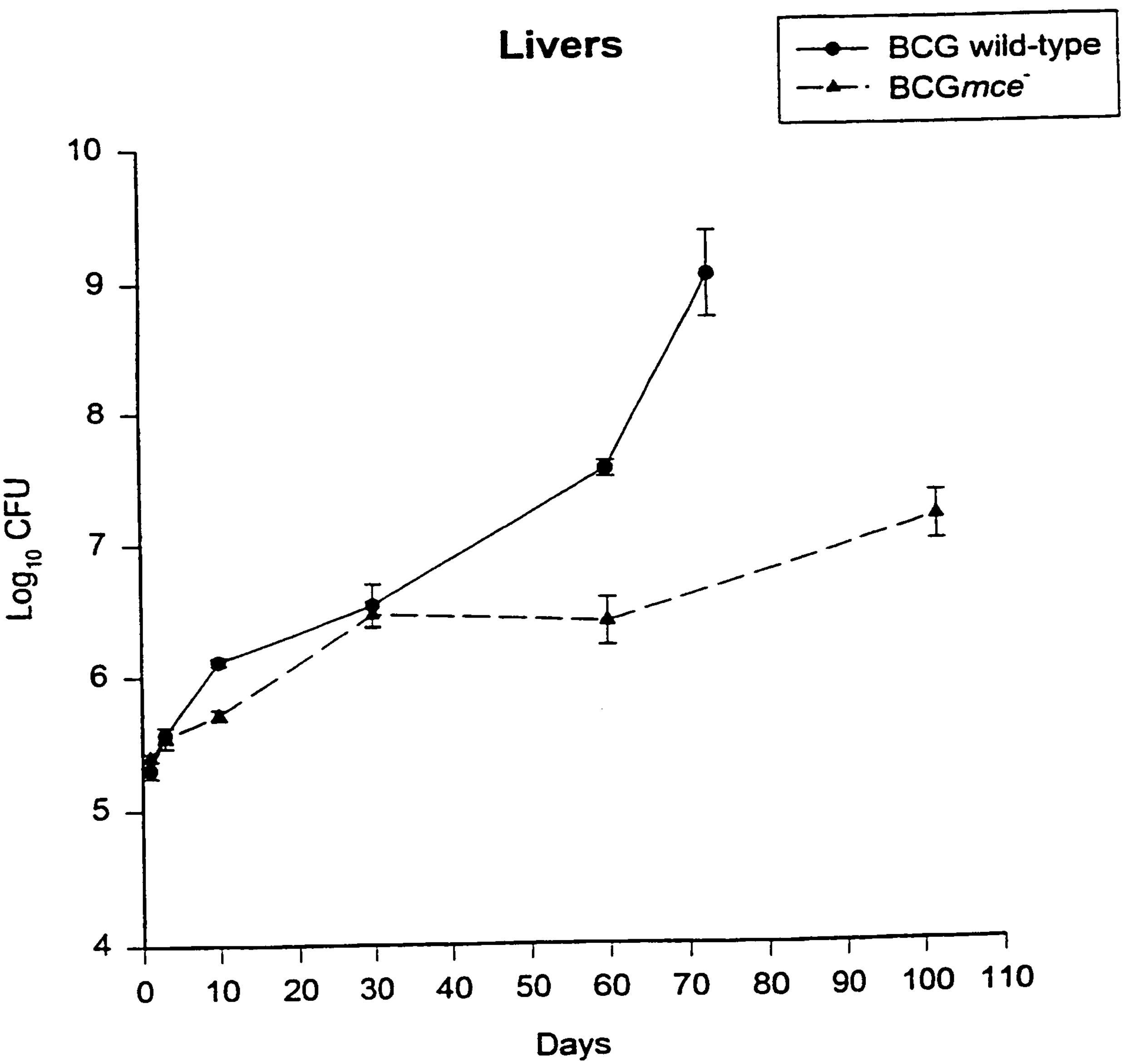

0.8
0.6
0.4
0.2
0.0
Percentage of invasion
BCG wild-type
BCGmce-
BCG Strain

FIG.5

FIGURE 6


Livers
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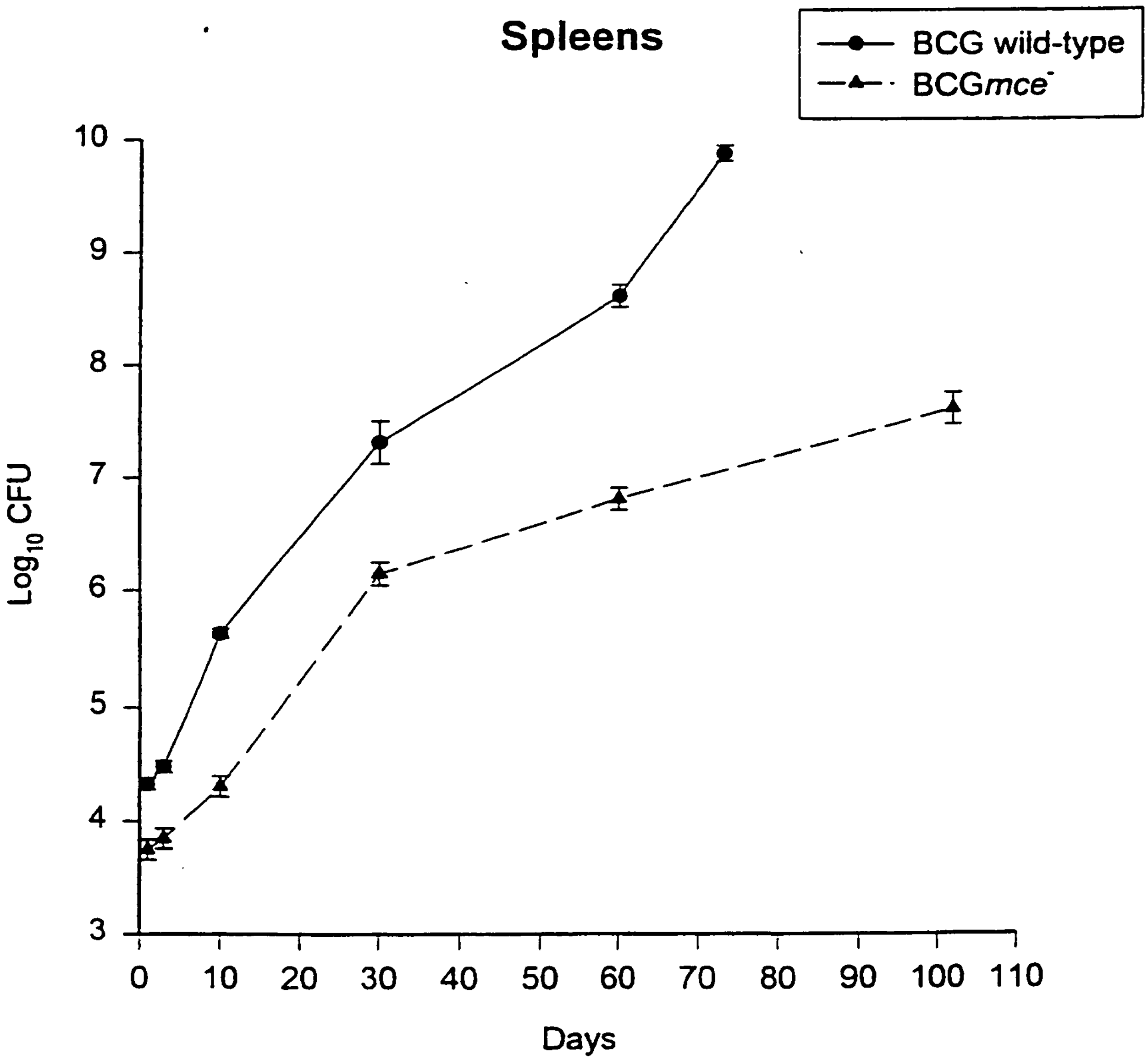
BCGmce⁻
10
9
8
7
6
5
4
Log10 CFU
0
10
20
30
40
50
60
70
80
90
100
110
Days

FIGURE 7


Spleens
BCG wild-type
BCGmce⁻
Log10 CFU
10
9
8
7
6
5
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3
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10
20
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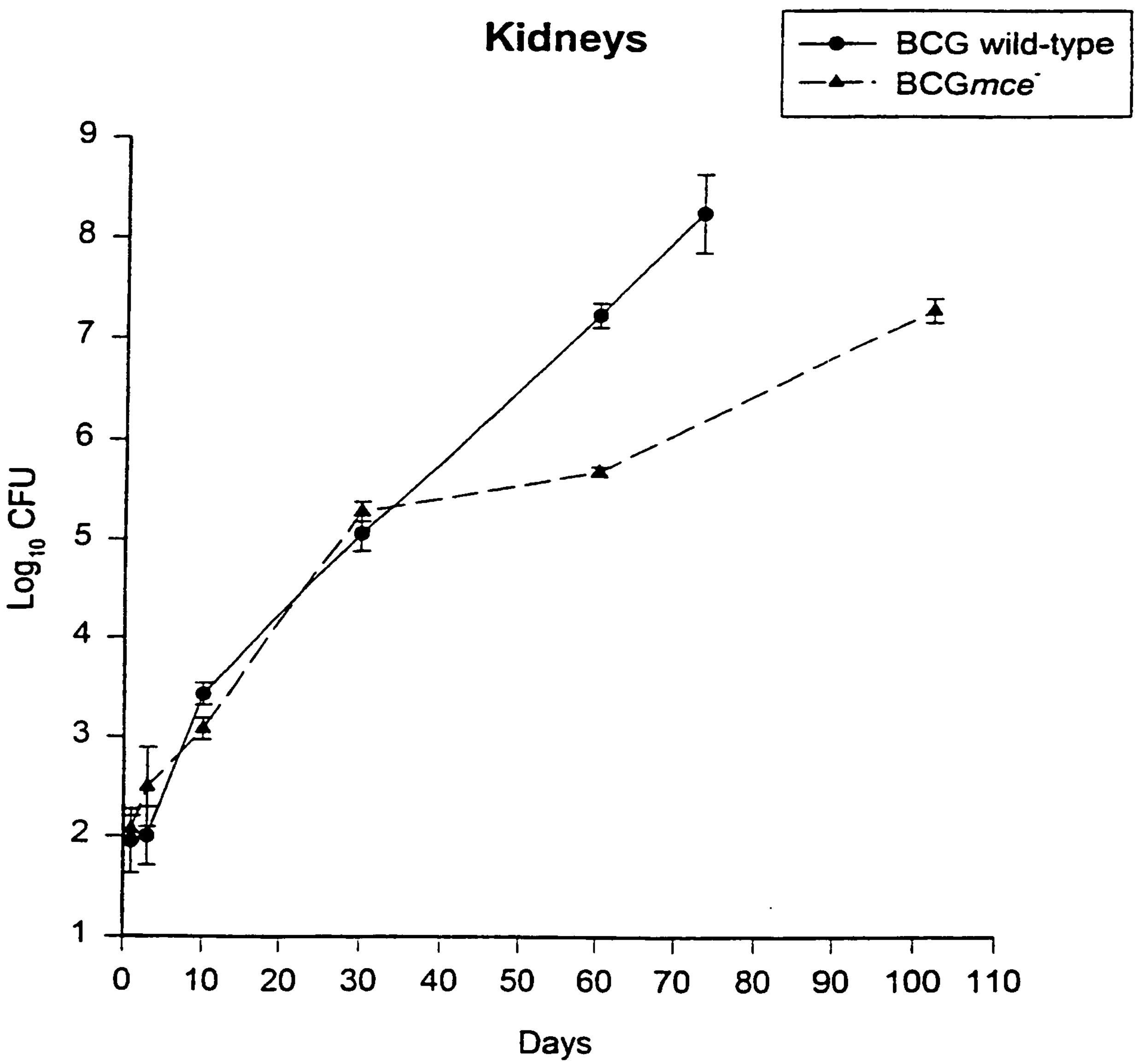
90
100
110
Days

FIGURE 8


Kidneys
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BCGmce⁻
Log10 CFU
9
8
7
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1
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10
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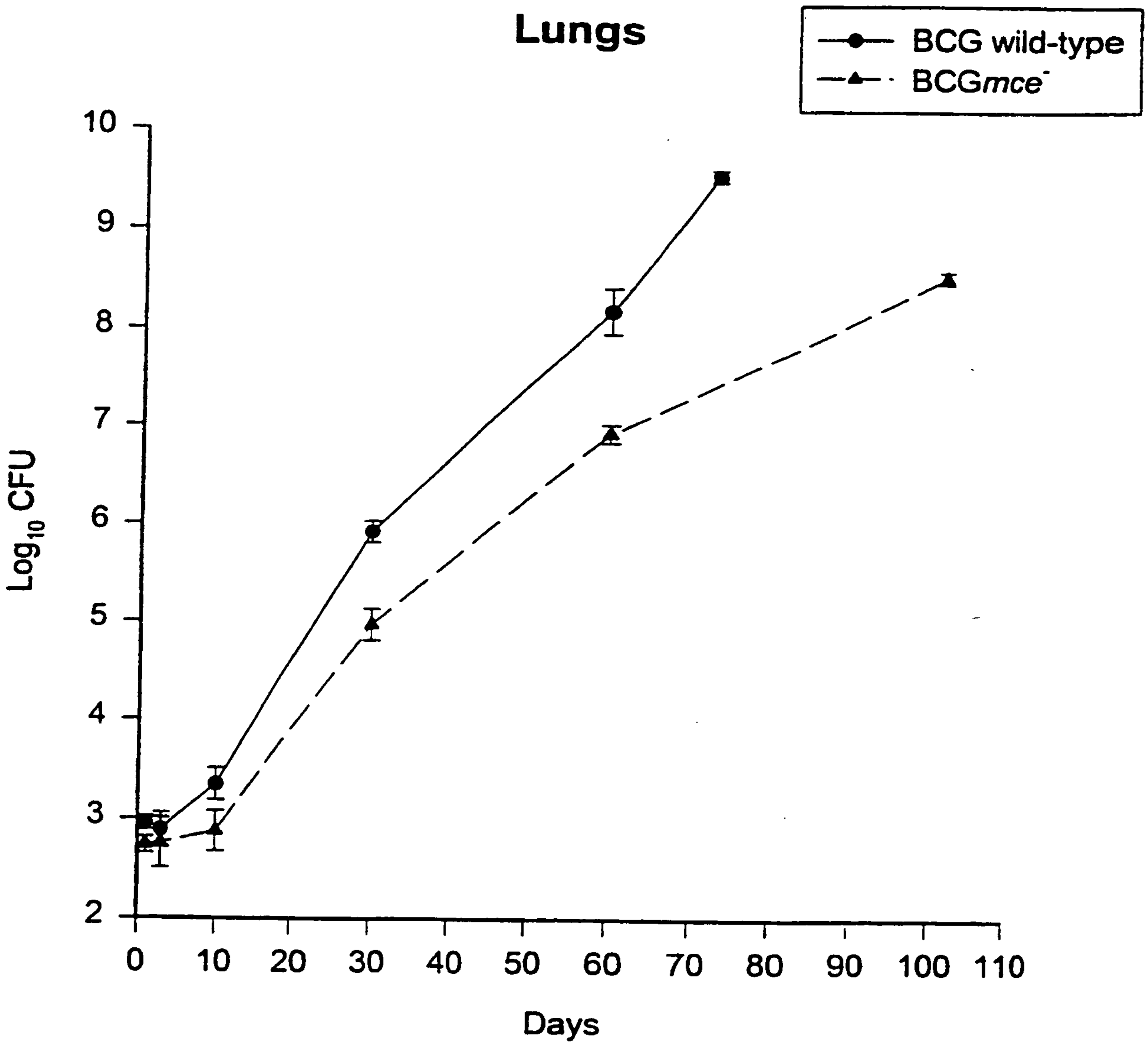
90
100
110
Days

FIGURE 9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BCGmce⁻
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8
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6
5
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40
50
60
70
80
90
100
110
Days

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 1/20, 15/31, A61K 39/04 // (C12N 1/20, C12R 1:32)

A3

(11) International Publication Number: WO 99/10475

(43) International Publication Date: 4 March 1999 (04.03.99)

(21) International Application Number: PCT/CA98/00790

(22) International Filing Date: 20 August 1998 (20.08.98)

(30) Priority Data:
08/915,709 21 August 1997 (21.08.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 08/915,709 (CIP)
Filed on 21 August 1997 (21.08.97)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report: 20 May 1999 (20.05.99)

(54) Title: ATTENUATED STRAINS OF MYCOBACTERIA

(57) Abstract

Attenuated strains of *Mycobacterium*, particularly species of the tuberculosis complex, have the mycobacterial cell entry (*mce*) gene functionally disabled. The gene may be disabled by an insertion into the gene which disrupts the mycobacterial cell entry function thereof of a selectable marker which is used for screen for homologous recombinants in which a double cross–over event has been effected. The attenuated strains may be used in the immunization of hosts against *Mycobacterium* disease.

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MD	Republic of Moldova
MG	Madagascar
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ML	Mali
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SE	Sweden
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SI	Slovenia
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SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
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ZW	Zimbabwe

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/CA 98/00790

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N1/20 C12N15/31 A61K39/04 //(C12N1/20,C12R1:32)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C12R C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 06726 A (CORNELL RESEARCH FOUNDATION) 9 March 1995 see page 4, line 23 - page 7, line 8; claims ---	1-30
A	WO 95 17511 A (AGRESEARCH NEW ZEALAND PASTORAL AGRICULTURE RESEARCH INSTITUTE, ET AL.) 29 June 1995 cited in the application see page 47, line 1 - line 11; claims 19-21 ---	1-30
P,A	WO 98 01559 A (CONNAUGHT LABORATORIES LIMITED) 15 January 1998 cited in the application see page 3, line 5 - page 9, line 24; claims -----	1-30

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 98/00790

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 22-28 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 98/00790

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